Regulation of Human Neurotropic JCV in Colon Cancer Cells

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Abstract. Background: Recently, the genome of the human polyomavirus, JC (JCV), and expression of its early and late regulatory proteins (T-antigen and agnoprotein) have been demonstrated in neoplastic cells of colonic cancer cases. Materials and Methods: Regulation of JCV was investigated in a human colon cancer cell line (SW480) and compared to a human glioblastoma cell line (U87-MG) that is permissive for JCV replication. Results: SW480 cells supported basal transcription of both early and late JCV promoters. The expression of TCF-4, a component of Wnt signaling, modulated JCV transcription in a cell type-specific manner. Both TCF-4 and T-antigen bound to the JCV promoter region and bound to each other. In addition, the expression of TCF-4 caused a decrease in the ability of the T-antigen to stimulate viral DNA replication in U87-MG cells. Conclusion: Wnt pathway signaling proteins and T-antigen interact to regulate JCV in colonic epithelial cells.

Studies have demonstrated an association between JCV and a broad range of human tumors including medulloblastoma (8-10), different types of glial brain tumor (11, 12), esophageal cancer (13) and colorectal carcinomas (14-16). JCV is capable of transforming cells and induces phenotypic alterations such as morphological changes, rapid division, prolonged lifespans as well as the ability to form dense foci in culture (17, 18). The oncogenic potential of this virus is not fully understood, but it is believed that a large part of its transforming activity involves its early regulatory protein, large T-antigen. The T-antigen, through interaction with several cell regulatory proteins including tumor suppressors, cell cycle regulators and transcription factors, promotes uncontrolled progression of cells through the cell cycle (19, 20). The potent oncogenic properties of the T-antigen have been established in several experimental animals (for review see Reference 21). In addition to interacting with the major tumor suppressors, p53 and pRb, it has also been demonstrated that JCV T-antigen has the potential to interact with several other signaling pathways (reviewed in Reference 22). These include the IGF-I (23) and Wnt (24) pathways in cells derived from JCV-induced mouse medulloblastoma. The Wnt pathway is important in colon carcinogenesis, as will be discussed below.

Surveys of raw sewage have revealed the presence of JC viral particles in sewage samples from widely diverse areas (3-6), while the detection of JCV in the urine of 20% to 80% of adults (25), plus studies demonstrating the presence of the JCV early regulatory protein, T-antigen, in esophageal (13) and colorectal carcinomas (14-16) suggest a potential re-entry of JCV and/ or viral DNA into the human population through the intake of virus-contaminated water and food. In support of this notion, studies have revealed the presence of JCV DNA sequences in the upper and lower human gastrointestinal (GI) tract (16, 26). The potential association of JCV with cancers of the GI tract is indicated by the expression of the early regulatory protein, T-antigen, and the late regulatory protein, agnoprotein, in some cases of esophageal (13) and colorectal cancer (14-16).
Dysregulation of the Wnt-signal transduction pathway is thought to be an early genetic event in the process of colorectal carcinogenesis (27). Such dysregulation of Wnt signaling has also been observed in experimental medulloblastomas from mice that were transgenic for the JCV early region encoding the T-antigen (24). In clonal T-antigen-positive cells from the explanted medulloblastoma (compared to T-antigen-negative cells), Western blot showed higher levels of β-catenin and LEF-1. Also, in JCV-positive clinical colon cancer samples, the nuclear detection of β-catenin and TCF-4 in T-antigen-positive cells suggests dysregulation of the Wnt pathway in these neoplastic cells (14). Further, experiments in tissue culture have indicated that the JCV T-antigen is able to bind directly to β-catenin and cause the deregulation of the Wnt pathway leading to the transcriptional activation of genes that promote cellular proliferation, e.g., c-myc (28).

Since JCV has been found to be present in some cases of colorectal cancer and is capable of disrupting Wnt signaling, we have now further investigated the regulation of JCV in colorectal cancer cells. For our studies the colorectal cancer cell line, SW480 was used and, as a control, the glioblastoma cell line, U87-MG, which is permissive for JCV transcription and replication. The regulation of JCV in these two cells and the relative effects of the T-antigen and the Wnt signal transducer, TCF-4, were compared.

Materials and Methods

Cell culture, transfection and plasmids. U87-MG human glioblastoma and SW480 human colon carcinoma cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and were transfected using the calcium phosphate precipitation method (29). The plasmid pcDNA3/zeo/JCVT expresses JCV T-antigen under the control of the CMV promoter has been described previously (23). All the GST-T-antigen constructs have been described previously (30). pcDNA3.1-β-catenin and pcDNA-TCF-4 have been described previously (31). The reporter constructs, JCVE-CAT and JCVI-CAT, contained the JCV promoter from the Mad-I strain linked to the chloramphenicol acetyltransferase (CAT) gene in the early and late orientations, respectively (32).

Antibodies. The antibody to TCF-4 was a goat polyclonal antibody (Santa Cruz Biotechnology, clone N-20), while the antibody to the T-antigen was a mouse monoclonal antibody (Oncogene Science, clone pAb416).

Transient transfection assays. U87-MG cells and SW480 cells were transfected by the calcium phosphate precipitation method (29) with reporter constructs alone (5 µg) or in combination with β-catenin, T-antigen and TCF-4 expression plasmids (5 µg). The total amount of DNA transfected into the cells was normalized with the relevant empty vector DNA. A glycerol shock was applied at 4 h post-transfection and the cells were harvested after 36 h. The chloramphenicol acetyl transferase (CAT) activity of the samples was determined by utilizing 100 µg of protein for each sample as previously described (30).

GST pull-down and Western blotting. GST pull-down assays were performed as we have previously described (30). Ten µg of either GST alone, GST-T-antigen, or its deletion mutant plasmids were diluted 1:10 in fresh Luria-Bertani broth supplemented with ampicillin (100 µg/ml). The cultures were induced with 0.3 M isopropyl–thiogalactopyranoside (IPTG) and GST fusion proteins were purified as previously described (30).

Co-immunoprecipitation and Western blot analysis. Co-immunoprecipitation and Western blot assays were performed as we have previously described (30). T-antigen and TCF-4 expression plasmids were transfected into SW480 and U87-MG cells via the calcium phosphate precipitation method (29). At 36 h post-transfection, the cells were lysed in lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.4) and 0.25% Nonidet P-40 supplemented with a cocktail of protease inhibitors including 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 µg/ml), aprotonin (1 µg/ml) and 50 mM sodium fluoride. Five hundred µg of whole-cell extract in a total volume of 0.5 ml was incubated with anti-T-antigen antibody or pre-immune antiserum overnight at 4°C. The immunocomplexes were precipitated with the addition of protein A-Sepharose beads (Pharmacia) for an additional 2 h followed by extensive washing with lysis buffer. The immunocomplexes were then resolved by SDS-10% PAGE and analyzed by Western blotting using anti-T-antigen antibody and developed with an ECL detection kit (Amersham, Arlington Heights, IL, USA) according to the manufacturer’s recommendations.

Expression and purification of recombinant GST fusion proteins. Fifty-milliliter overnight cultures of Escherichia coli DH5 transformed with pGEX2T-JCV T-antigen or its deletion mutant plasmids were diluted 1:10 in fresh Luria-Bertani broth supplemented with ampicillin (100 µg/ml). The cultures were induced with 0.3 M isopropyl–thiogalactopyranoside (IPTG) and GST fusion proteins were purified as previously described (30). The fusion proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining.

Double labeling immunofluorescence. SW480 and U87-MG cells were transfected with 5 µg of pcDNA3/zeo/JCVT and 5 µg of pcDNA-TCF-4 by the calcium phosphate precipitation method. The cells were washed with PBS, fixed in cold acetone and blocked in PBS containing 5% horse serum and 0.1% BSA for 2 h. The cells were then incubated with mouse anti-T-antigen antibody (1:100 dilution for 16 h). Following the 16-h-incubation the cells were washed in PBS and incubated in anti-mouse rhodamine antibody (Vector Labs, 1:200 dilution) for 1 h. Next, the cells were blocked for 2 h in PBS containing 5% rabbit serum and 0.1% BSA before incubation with anti-TCF-4 antibody (1:200 dilution for 16 h), followed by washing in PBS and incubation in anti-goat fluorescein antibody (Vector Labs, 1:200 dilution). The cells were then washed in PBS and mounted in aqueous mounting media (Vector Labs).
DNA-protein binding assay. For the DNA-protein binding assay, cells were transfected with the T-antigen and TCF-4 plasmid alone and together and the nuclear proteins were extracted as described previously (33). For immunoprecipitation, samples were incubated with anti-T-antigen, anti-TCF-4 or pre-immune serum as control and nick-translated 32P-labeled DNA containing the complete JCV non-transcribed control region. DNA from the immunocomplexes was separated on a 3% agarose gel and visualized by autoradiography.

ChIP assay. SW480 and U87-MG cells were transfected with plasmid containing the JCV genome (pBJC) alone or in combination with plasmids expressing the T-antigen and TCF-4, then chromatin immunoprecipitation (ChIP) was performed as we have previously described (34) using the ChIP assay kit (Upstate Cell Signaling Solutions). Briefly, cross-linking was performed with formaldehyde and the DNA sheared by sonication. The cells were lysed and immunoprecipitation was performed with antibodies as indicated. The following primers spanning the JCV control region were used for PCR: atcctccctattcagca and caaaacagctctggctcgcaa.

Colony formation assay. Transfection for the colony formation assay was carried out as described for Western blot analysis with the T-antigen and TCF-4 plasmid alone and together. As controls, cells were also transfected with pcDNA3.0 (with the neomycin resistance gene) and pcDNA3.1 (with the zeocin resistance gene) plasmids. Following the transfections in 60-mm dishes, the cells were trypsinized and replated into six 100-mm dishes from each 60-mm dish. The cells were then treated with G418 and Zeocin, alone or together, for 3 weeks on an every other day schedule. After 3 weeks, the dishes were washed and incubated with methylene blue dye for 2 min and washed with running water. The dishes were then dried overnight and the colonies were counted.

Effect of TCF-4 on viral DNA replication. SW480 and U87-MG cells were transfected with 5 µg of the replication-competent JCVp543CAT plasmid alone or with 5 µg of pcDNA3.1-T-antigen and pcDNA3.0-TCF-4 plasmids. Low molecular weight (LMW) DNA was collected in 10 mM Tris, 10 mM EDTA and 0.6% SDS (Hirt) buffer, as we have described previously (30). The LMW DNA was digested with EcoRI and DpnI enzymes for 6 h at 37°C. Following the enzymatic digestion, DNA was purified using phenol-chloroform extraction and run on a 1% agarose gel. The DNA from the gel was then transferred to a Hybond N membrane and cross-linked. The membranes were then hybridized overnight with a [32P]-labeled denatured DNA probe corresponding to the JCV early promoter.

Results

Earlier studies indicated that the JCV T-antigen disrupts Wnt signaling in medulloblastoma and colon carcinoma cells (14, 24, 28). The expression of the JCV T-antigen has been detected in clinical cases of colon carcinoma (14-16). The present study investigated a mechanism by which JCV is regulated in the colonic epithelial cell line, SW480, a colorectal cancer cell line. Experiments were also conducted in parallel with a human glioblastoma cell line, U87-MG, which is known to be permissive for JCV gene expression and replication.

In Figure 1, it is shown that the SW480 and U87-MG cell lines are both capable of supporting basal levels of JCV early and late promoter transcription. The human colon cancer cell line, SW480 (A), and human glioblastoma cell line, U87-MG (B), were transfected with 5 µg of a plasmid containing the JCV early or late promoter upstream of a CAT reporter.
gene, either alone or together with plasmids expressing the JCV T-antigen or the cellular proteins β-catenin and TCF-4, which are key regulators of the Wnt pathway. The level of CAT activity was measured after 36 h. In SW480, the T-antigen significantly increased the transcription of the JCV early promoter (Panel A, compare lanes 1 and 3). The effect of the T-antigen seen on the JCV early promoter was greatly decreased by TCF-4 (Panel A, compare lanes 3 and 6). While the T-antigen significantly increased the transcription of the early promoter, it only modestly stimulated the late promoter activity (Panel A, compare lanes 7 and 9). Different effects of the T-antigen and TCF-4 are seen in U87-MG transfected with the promoters and the plasmids. Modest stimulation of transcription of the early and late gene is seen with T-antigen (Panel B, compare lanes 1 and 3 and lanes 7 and 9). In U87-MG cells, β-catenin and TCF-4 both strongly stimulated JCV early gene expression (Panel B, compare lanes 1 with 2 and 4), but not in SW480 cells (Panel A, compare lanes 1 with 2 and 4). Thus, the effects of ectopic T-antigen and TCF-4 expression on the expression of JCV promoters are dependent on cell type. Results with standard errors are given for the CAT assays for each cell line with a representative experiment shown as an inset. All the CAT assays were repeated three times.

The activities of the JCV early and late promoters are both controlled by the same DNA region of the JCV genome (the non-transcribed control region), which is inserted in reverse orientation with respect to the reporter gene in the late promoter construct compared to the early reporter constructs. Since the T-antigen and TCF-4 can modulate early and late promoter activities, next the ability of the T-antigen and TCF-4 to bind to this DNA region was determined. This binding was measured either in cell extracts using a DNA-protein binding assay or in live cells using a ChIP assay (Figure 2). Panel A demonstrates DNA-protein binding experiments with cell extracts from transfected SW480 and U87-MG cells and radiolabeled JCV promoter DNA probe. In each case, the DNA band shown was immunoprecipitated by the addition of anti-T-antigen antibody or control pre-immune serum. An immunoprecipitated band was seen for both T-antigen (lanes 1 and 10) and TCF-4 (lanes 4 and 7), indicating that both proteins are capable of binding to the JCV promoter region. In Panel B, the binding of these proteins in vivo was investigated using the ChIP assay. Cells were transfected with plasmid containing the complete JCV genome plus plasmid expressing the T-antigen and/or TCF-4 as indicated. Again binding to the JCV promoter was demonstrated for the T-antigen (lanes 1 and 10) and TCF-4 (lanes 4 and 7). The same pre-immune antibodies were employed as negative controls and input cell extracts were used as positive controls (marked with *).

Since the T-antigen and TCF-4 are able to modulate each other’s effects on transcription of the JCV promoters, we reasoned that they might physically interact with each other. To investigate this, whether there was a physical association between the T-antigen and TCF-4 was determined using a co-immunoprecipitation/Western blot approach. SW80 and
U87-MG cells were transfected with plasmids expressing the T-antigen and/or TCF-4 as indicated (Figure 3). Panel A verifies the expression of the T-antigen and TCF-4 in the transfected SW480 and U87-MG cells. Panel B shows that JCV T-antigen and TCF-4 physically interacted with each other, as determined by immunoprecipitation with antibody against the T-antigen followed by a Western blot with antibody against TCF-4. In cells transfected with the T-antigen, a band corresponding to TCF-4 was co-immunoprecipitated with antibody to the T-antigen but not with pre-immune serum (Figure 3B, compare lanes 2 and 6).

Next, GST pull down assays were used to determine the region within the T-antigen protein that is responsible for binding to TCF-4. The interaction of TCF-4 and the T-antigen was mapped for the T-antigen protein using full length and mutant T-antigen fragments that were fused in frame with GST to create a series of GST-fusion proteins. These were then incubated with cell extracts from SW80 or U87-MG cells that had been transfected with plasmid expressing TCF-4. Figure 4 demonstrates the regions of the T-antigen to which TCF-4 is able to bind. The strongest interaction between the T-antigen and TCF-4 was seen with the mutant containing the 266 to 688 amino acid region. Interestingly, some differences were seen between the cell lines. For example, mutants 1-265 and 1-411 bound to TCF-4 in U87-MG but not in SW480 cells, whereas mutant 412-688 bound strongly to TCF-4 in SW480 but only weakly in U87-MG cells. This strongly suggests that one or more other cell type-specific proteins are involved in the interaction of the T-antigen with TCF-4 and this may also be related to the cell type-specific effects of these proteins on JCV transcription.

The subcellular localization of the T-antigen and TCF-4 was next investigated by immunocytochemistry. U87-MG and SW480 cells were transfected with 5 μg of T-antigen and TCF-4 plasmids using the calcium phosphate method and fixed with cold acetone 36 h after transfection. The transfected cells were then double-labeled with antibodies against the T-antigen and TCF-4, demonstrating that JCV T-antigen and TCF-4 co-localized in the nucleus (Figure 5).

To determine whether or not TCF-4 has other effects on the regulation of JCV in colonic epithelial cells, the following experiments were conducted. Figure 6 shows the results obtained from a DpnI replication assay that measures the ability of viral DNA to replicate. The DpnI replication assay utilizes the enzyme DpnI which digests DNA that is not protected by the methylation which occurs when DNA is newly replicated, i.e., input DNA is removed by digestion leaving a band corresponding to replicated DNA. Plasmid containing the JCV non-transcribed regulatory region (JCVE) was transfected into SW480 and U87-MG alone and in the presence of plasmids expressing the T-antigen and/or TCF-4. Low molecular DNA corresponding to replicated JCV was separated on an agarose gel after DpnI treatment and subject to Southern blot hybridization with a radiolabeled JCV-specific DNA probe. In both cell lines, the T-antigen was required for viral replication (compare lanes 1 and 2). It was seen that TCF-4 was capable of suppressing viral DNA replication in U87-MG but not SW480 cells (compare lanes 3 and 4). These observations again point to the cross-interaction of TCF-4 and the T-antigen and the involvement of cell type-specific proteins.

The data so far indicate that the T-antigen and TCF-4 cross-interact and that this involves one or more cell type-specific factors. It is also known that the T-antigen and TCF-4 may play a role in cell growth regulation and carcinogenesis. In the final set of experiments, whether...
T-antigen and TCF-4 expression in SW480 cells had any effect on their ability to form colonies was examined (Figure 7). Expression of TCF-4 stimulated colony formation by SW480 (compare lanes 1 and 2), while the T-antigen also caused some stimulation (compare lanes 5 and 6).

**Discussion**

The highly neurotropic JC virus is usually thought to be expressed only in astrocytes and oligodendrocytes of the human brain where it is responsible for the fatal demyelinating disease, PML. This tropism is believed to be due to a requirement for tissue-specific transcription factors that are restricted to glial cells (35) and may also reflect the involvement of tissue-specific cell surface proteins in the entry of JC virions into the cell (36). However, it is also clear that JCV can be found in a range of other human cell types and tissues. The JCV genome has been detected in tonsillar stromal cells (37), B lymphoid cells (38), kidney epithelial cells (39) and upper and lower parts of the GI tract, particularly the mucosa of the colon (15, 16, 26). Moreover, JCV genomic DNA and the expression of early and late regulatory proteins, T-antigen and agnoprotein have been detected in esophageal cancer (13) and colorectal cancer (14-16) clinical samples. Since most studies on JCV gene expression to date have focused on cells of glial origin, it was of interest to examine JCV in cells derived from the GI tract. To this end, JCV regulation in SW480 human colon carcinoma cells was compared to U87-MG human glioblastoma cells.

Both the early and late JCV promoters were found to be active in SW480 cells but their responses to the three transcription factors tested (T-antigen, β-catenin and TCF-4) were very different. For example, the late promoter was highly induced by β-catenin and TCF-4 in SW480 cells but not in U87-MG cells. It is known that the regulation of JCV gene expression is regulated by many transcription factors, some of which are general transcription factors that are expressed in all cell types and others that are specific for glial cells (40). The absence of the glial cell-specific transcription factors in SW480 cells may account for the differences observed in the regulation of the JCV promoters between SW480 and U87-MG cells. Alternatively, it is possible that cell type-specific transcription factors expressed in SW480 but not U87-MG cells modulated the transcription of JCV.

TCF-4 is a partner of β-catenin and is an integral part of the Wnt signaling pathway. Our results demonstrated that the T-antigen and TCF-4 can regulate JCV gene expression and can both bind to the JCV promoter, as determined by DNA binding and ChIP assays. Furthermore, IP/Western and GST
Figure 5. Co-localization of TCF-4 and T-antigen by immunocytochemistry. SW480 and U87-MG cells were transfected with the T-antigen expression plasmid and TCF-4 expression plasmid. The cells were fixed 48 h after transfection using 10% formalin. Immunocytochemical labeling was performed with antibodies to the T-antigen (left panels) and TCF-4 (center panels). Superposition of the double-labeled cells with anti-T-antigen and anti-TCF-4 is shown in the right hand panels.

Figure 6. Effect of expression of the T-antigen and TCF-4 on JCV replication. SW480 and U87-MG cells were transfected with 10 µg of a plasmid containing the JCV non-transcribed control region alone or together with plasmids expressing the JCV T-antigen and/or TCF-4 as indicated. Low molecular weight DNA was extracted from the transfected cells after 36 h and digested with the restriction enzymes BamH1 and DpnI at 37°C for 16 h to remove unreplicated input DNA. Digested samples were run on 1% agarose and analyzed by Southern blot to detect replicated JCV DNA.

Figure 7. Effect of T-antigen and TCF-4 expression on colony formation by SW480 cells. SW480 cells were transfected with the plasmid vector pcDNA3.1/Zeo+, pcDNA3.1/Zeo+ expressing T-antigen, vector pcDNA3.0/Neo+ and/or pcDNA3.0/Neo expressing TCF-4 as indicated. The transfected cells were then treated with Zeocin and/or G418. After 3 weeks, the cells were stained with methylene blue dye and colonies were counted. The experiment was repeated and the counts are presented with the standard error.
pull-down assays showed that the T-antigen and TCF-4 can bind to each other and immunocytochemistry demonstrated that both proteins colocalized to the nucleus. Interestingly, the mapping of the TCF-4 binding domain within the T-antigen protein using T-antigen deletion mutants demonstrated that some differences exist between SW480 and U87-MG cells with respect to the exact localization of this domain. This may be due to the involvement of other cell-specific proteins in the interaction between the T-antigen and TCF-4.

The TCF-4 binding motif ctttgtc is present in the control region sequence of JCV and the data presented here indicate that TCF-4 binds to the JCV promoter in vivo. Interestingly, TCF-4 inhibits T-antigen-dependent JCV DNA replication in U87-MG but not SW480 cells. Colon carcinoma cells from samples that are positive for JCV are not thought to be actively replicating the virus since they do not express the viral capsid proteins. However, these cells often express agnoprotein which, like the capsid proteins, is expressed from the late promoter. This suggests that the JCV genomic DNA present in these tumors may have lost the capsid genes or that some form of post-transcriptional control prevented them from being expressed at detectable levels.

Dysregulation of transcription triggered by Wnt signaling molecules contributes to colorectal carcinogenesis (27). β-catenin accumulation is often caused by mutations in the tumor suppressor APC which binds to β-catenin (41), this being an early step in oncogenesis for many colorectal cancers. Mutations of APC cause aberrant accumulation of β-catenin which then binds TCF-4 in the nucleus where it stimulates the transcription of growth promoting genes, e.g., c-myc (42). This is consistent with the data in Figure 7 where ectopic expression of TCF-4 caused enhanced colony formation by S480 cells. Previously we reported that the JCV T-antigen and β-catenin stimulate the activity of the promoter for c-myc and a synthetic TCF reporter plasmid (28). In this study, the T-antigen was shown to be present as a complex in association with β-catenin, and this complex may be involved in the regulation of genes such a c-myc and other Wnt-responsive growth-promoting genes and promote cell proliferation in colon carcinoma cells that express the JCV T-antigen.

In conclusion, the results presented in this manuscript extend earlier reports on the interaction of the JCV T-antigen and Wnt signaling molecules. These observations should invite further investigation into the association of the JCV T-antigen and TCF-4 in human colorectal cancer. The development of animal models for human colorectal cancer using JCV as a tool and further investigations of the pathways of cell signaling by JCV may shed new light on the multiple steps of deregulation that lead to the development of colorectal carcinoma.

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